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PRINCIPAL INVESTIGATOR: Conor C. Lynch, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University
Nashville, TN, 37232

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Conor C. Lynch, Ph.D.

E-Mail: conor.lynch@vanderbilt.edu

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14. ABSTRACT
Using an animal model of prostate tumor progression in the bone we have previously shown that MMPs, namely MMP-2,-3,-9 and -13, are overexpressed at the tumor bone interface and these MMPs are for the most part expressed by the host cells of the bone. To test the contribution of MMPs in prostate tumor progression in the bone, we have generated mice that are immunocompromized and deficient for MMP-2,-3 and -9 during the current period. We have found that MMP-9 does not contribute to prostate tumor progression in the bone since no difference in osteolytic or osteoblastic responses between wild type and MMP-9 deficient animals were detected by Faxitron, CT, SPECT and histomorphometry. These results, while negative, are important for the generation of selective MMP inhibitors that lack the deleterious side effects associated with broad spectrum inhibitors. In addition, we have also identified PTHrP as an MMP substrate and postulate that MMP processing of PTHrP may be a mechanism through which MMPs can contribute to tumor induced osteolysis.

15. SUBJECT TERMS
Osteolysis, osteoblastic changes, prostate progression in bone, matrix metalloproteinases, MMPs, receptor activator of nuclear kappa B ligand, RANKL, bone metastasis

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Introduction…….…..

This year, in the United States alone, of the 27,350 men who die from prostate cancer, 80% will have evidence of bone metastasis [1, 2]. Prostate bone metastases cause several complications for patients such as hypercalcemia, spontaneous bone fracture and debilitating pain that dramatically affects their quality of life. To progress in the bone, the invading prostate tumor cells induce radical changes in bone matrix homeostasis by stimulating osteoblastic and osteolytic changes [3]. These changes result in an actively remodeling bone tumor microenvironment, rich in mitogenic signals that promote tumor growth. In turn, the growth of the tumor exacerbates the osteoblastic and osteolytic changes in a manner that has been well described as the 'vicious cycle' [4]. Using an animal model of tumor progression in the bone, we have previously identified a group of enzymes known as matrix metalloproteinases (MMPs) as being highly overexpressed at the tumor bone interface in comparison to the tumor area alone. In a bid to understand the importance of these MMPs, namely MMP-2, -3, -9 and -13, in prostate tumor progression in the bone, we aim to generate MMP null animals and compare those animals to their wild type counterparts. While the MMPs are important in the turnover of the

extracelullar matrix, it has become apparent that the MMPs are also capable of regulating cell:cell communication by processing various cytokines and growth factors to active soluble forms [5]. These soluble factors often influence biological processes including survival, proliferation, angiogenesis and osteoclast activation. Therefore, understanding which MMPs are important in contributing to prostate tumor progression in the bone and identifying the mechansisms that govern the vicious cycle can provide valuable targets for therapeutic development.

Body……..

Accomplishments

Aim 1. *Determine the stromal contribution of MMPs that are markedly overexpressed at the tumor:bone interface namely, MMP-2,-3,-9 and -13 to prostate cancer induced osteoblastic and osteolytic changes in the bone*.

a) Generate immunocompromised RAG-2$^{-/-}$ mice that are deficient in MMP-2, MMP-3 and MMP-13 by crossing RAG-2$^{-/-}$ mice with MMP$^{-/-}$ mice that are both available on the C57Bl/6 background (Months 1-12).
b) Using our pre-clinical animal models, we will test the contribution of stromal MMP-9 to tumor induced osteoblastic and osteolytic change in readily available immunocompromised RAG-2$^{-/-}$ MMP-9 deficient mice (Months 1-12).
c) Test the contribution of stromal MMP-2, MMP-3 and MMP-13 to tumor induced osteoblastic and osteolytic change using our pre-clinical model (Months 11-30).
d) Identify the expression of stromal MMPs in human clinical samples of prostate bone metastases (Months 20-36).

The proposed animal model in the current program involves the transplantation of moderately differentiated rat prostate adenocarcinoma to the calvaria of immunocompromized wild type and MMP deficient mice. To achieve this, we proposed to cross C57Bl/6 RAG-2 (recombinase activating gene-2) deficient mice with either C57Bl/6 MMP-2, -3 or -13 deficient animals in order to generate F2/F3 animals that are immunocompromized and deficient for the desired MMP. As of December 2007, we have generated RAG-2$^{-/-}$;MMP-2$^{-/-}$ and RAG-2$^{-/-}$;MMP-3$^{-/-}$ animals and are currently generating enough mice in order to perform our proposed studies. The

MMP-13 null mice on a C57Bl/6 background were obtained from Dr. Steven Krane, Harvard, Boston, MA and we are currently generating RAG-2$^{-/-}$;MMP-13$^{-/-}$ null animals.

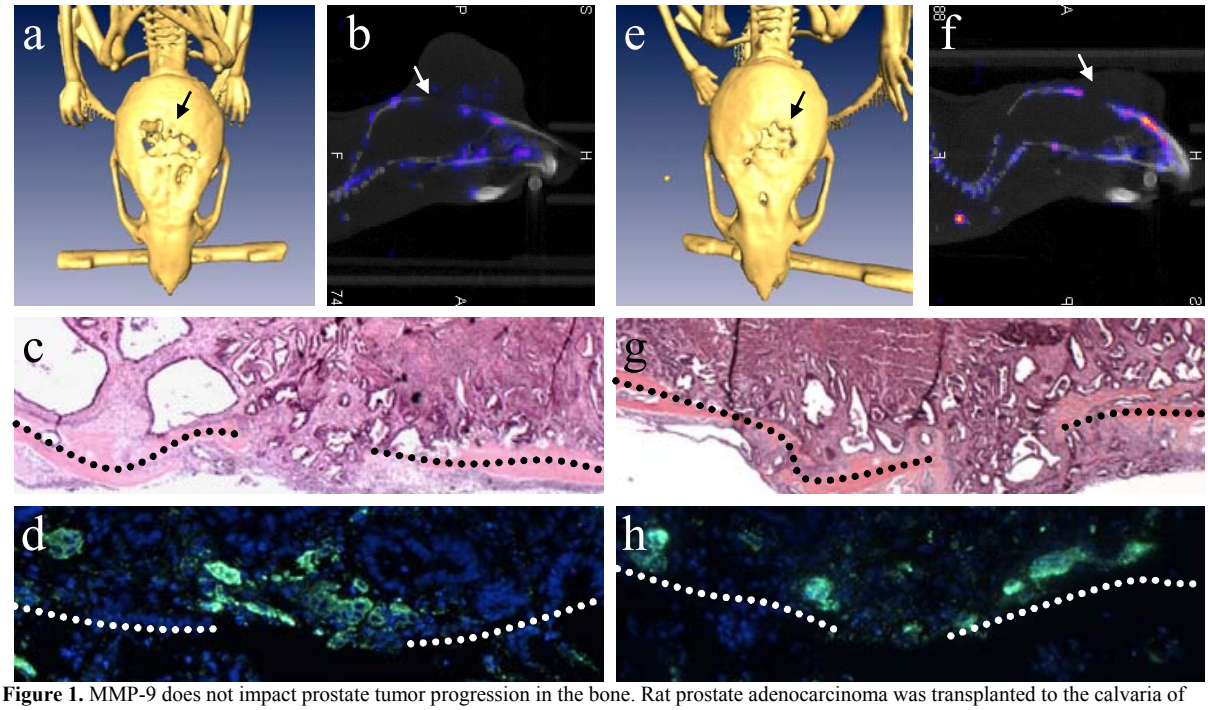

Figure 1. MMP-9 does not impact prostate tumor progression in the bone. Rat prostate adenocarcinoma was transplanted to the calvaria of age matched wild type (a-d; n=10) and MMP-9 null (e-h; n=13) male immunocompromized mice. X-ray (data not shown), microCT (a vs. e) and microspect with Tc-MDP99 (b vs. f) showed no gross difference in the amount of tumor induced osteoblastic or osteolytic (arrows) activity between the wild type and MMP-9 null mice. Histological analysis also revealed that there was no significant difference in the amount of bone loss by H&E (c vs. g; bone represented by dashed lines) or in the number of osteoclasts at the tumor bone interface (d vs. h; TRAP positive osteoclasts in green with nuclei in blue).

RAG-2;MMP-9 null animals had been generated previously and in the past year, the impact of host MMP-9 on prostate tumor progression was tested. In repeated studies, with at least 10 animals per group, we determined that MMP-9 does not have any effect on tumor progression in the bone. While there is a trend towards a decrease in osteolysis in the MMP-9 deficient animals, this decrease has not proven to be statistically significant (Figure 1). While these data suggest that MMP-9 does not contribute to tumor progression in the bone, it should be stated that the role of MMP-9 in 1) the metastasis of prostate cancer to the bone or 2) in the initial survival/establishment of the prostate tumor cells in the bone microenvironment can not be ruled out since these steps are not recapitulated in our animal model. Several reports have documented the contribution of host derived MMP-9 to angiogenesis and therefore, we will examine if there is a difference in blood vessel number between the wild type and knockout animals [6]. This

finding relating to MMP-9 and tumor progression is in keeping with a published report using a human prostate tumor cell line, PC-3 in a RAG-1;MMP-9 null animal [7]. We are currently preparing a manuscript documenting our findings.

To determine the clinical relevance of host MMPs in tumor progression in the bone, we have been collecting non-identified samples of human prostate to bone metastasis. Currently, we have collected 2 cases.

Aim 2. *Identify and test MMP processed substrates that mediate prostate tumor induced osteolytic and osteoblastic change.*

a) Identify and test candidate MMP substrates that mediate prostate tumor induced osteolytic and osteoblastic change.
b) Determine the contribution of MMP solubilized RANKL vs. MMP resistant RANKL in mediating osteoclastogenesis

In aim 2, we have taken a candidate approach in a bid to identify the potential factors that MMPs process in order to mediate tumor induced osteolysis. Bone is a rich reservoir of growth factors such as transforming growth factorβ (TGFβ) and insulin like growth factor-1 (IGF-1) and both of these factors have been implicated in driving the 'vicious cycle' [4]. Given that various MMPs have been reported as processing the molecules that keep these growth factors in a latent state such as latency TGFβ binding proteins (LTBPs) and IGF binding proteins (IGF-BPs) we envisage that these would be excellent candidate molecules through which MMPs could contribute to osteolytic and osteoblastic effects. Using ELISA, we have observed that there is more active TGFβ at the tumor bone interface in comparison to the tumor area alone in wild type animals (Figure 2). Using this approach and following up with western blot and immunolocalization studies, we have the ability to rapidly

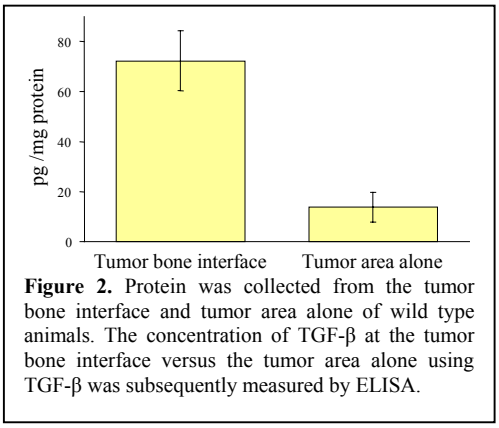

Figure 2. Protein was collected from the tumor bone interface and tumor area alone of wild type animals. The concentration of TGF-β at the tumor bone interface versus the tumor area alone using TGF-β was subsequently measured by ELISA.

assess if the absence of host derived MMPs, such as MMP-2, -3 and -13 are playing a role in the release of active TGFβ from the bone.

In the metastatic bone:tumor microenvironment, parathyroid related hormone (PTHrP) has been identified as a powerful mediator of osteolysis [8]. Pro-PTHrP has three isoforms that are 139, 141 or 173 amino acids in length. These isoforms are subsequently enzymatically processed to yield the mature form of $PTHrP_{1\text{-}36}$ (amino acids 1-36). Thus far the enzymes implicated in generating mature PTHrP have been; endothelin converting enzyme-1 (ECE-1); ECE-2 and neprilysin which are not MMPs but are members of the metazincin family of proteinases. Interestingly, prostate specific antigen (PSA) which is a serine protease has also been shown to process PTHrP but in a different region that generates a 23 amino acid form of $PTHrP_{1\text{-}23}$ (Cramer et al., 1996). This is thought to abolish the activity of the hormone but some studies suggest that smaller molecular weight versions of PTHrP can have differential effects compared to $PTHrP_{1\text{-}36}$ [9].

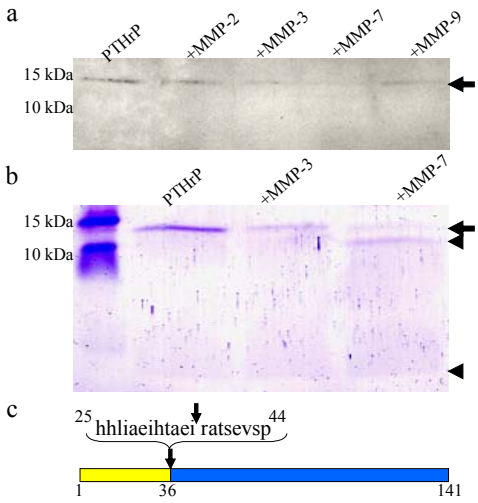

Figure 3. MMP-3 and MMP-7 process PTHrP (amino acid 1-141) to a mature form. **A.** Mature PTHrP (100ng; arrow) was incubated for 1 hour at 37oC either alone or with 100ng of active MMP-2, -3, -7 and -9. Using an antibody directed to the full length form of PTHrP, western blot analysis revealed that MMP-3 and MMP-7 were capable of processing PTHrP (arrow heads). **B-C.** To identify the site of cleavage, the PTHrP bands generated by MMP-3 and MMP-7 were blotted onto a nitrocellulose membrane, excised and subjected to N-terminal amino acid sequencing. Results show that PTHrP was processed at amino acid 37 which would generate a putative mature active form of PTHrP (1-36). Arrow in C represents site of MMP cleavage; numbers represent amino acid position.

Since PTHrP can be processed by members of the metazincin family and given the presence of MMPs in the tumor bone microenvironment, we asked whether MMPs could process PTHrP. Using recombinant $PTHrP_{1\text{-}141}$, we observed that MMP-3 and MMP-7 generate mature $PTHrP_{1\text{-}36}$ (Figure 3). We are currently using mass spcectroscopy to identify the presence of other cleavage products. These data indicate that host derived MMP-3 and MMP-7 may play a role in promoting tumor induced osteolysis via the processing of pro-PTHrP to a mature form.

We have previously demonstrated that membrane bound receptor activator of nuclear κB ligand (RANKL) which is essential for osteoclast maturation and activation is sensitive to shedding from the cell surface by MMP-3 and MMP-7 [10]. We have generated a non-cleavable version of RANKL and are currently testing the ability of the non-cleaved RANKL to stimulate osteoclast activation via direct cell:cell contact.

Key Research Accomplishments……..……..

- Generated RAG-2;MMP-2 and RAG-2;MMP3 null animals
- Observed that host derived MMP-9 does not contribute to tumor progression in the bone
- Have begun the collection of human samples of prostate to bone metastasis
- Identified higher levels of TGFβ at the tumor bone interface in comparison to the tumor area alone in wild type animals
- Identified that MMP-3 and MMP-7 are capable of generating mature PTHrP
- Have generated a non-cleavable version of RANKL

Reportable Outcomes……

Manuscripts

Halpern, JL., Tawtawny, MN., and Lynch, CC. Host matrix metalloproteinase-9 does not impact tumor induced osteolytic/osteoblastic changes in a model of prostate cancer bone metastasis. Manuscript in preparation.

Presentations

The impact of host derived MMPs in tumor induced osteolysis. Tumor host interaction and angiogenesis meeting, Monte Verita, Ascona, Switzerland, October 2007.

TGFβ and the tumor: bone microenvironment. Tumor microenvironment (TMEN) meeting, Vanderbilt University, Nashville, TN, September, 2007.

Conclusion……

In previous studies, we have identified that MMPs, namely MMP-2, -3, -9 and -13 are overexpressed at the tumor: bone interface. In the past, human clinical trials involving broad spectrum MMP inhibitors failed due to dose limiting side effects. The principle reason for the failure was due to a lack of understanding as to how MMPs contribute to tumor progression. In animals models of osteolysis, broad spectrum MMP inhibitors have been successful in preventing tumor induced osteolysis and growth (REF). Therefore, in order to apply MMP inhibitors in the clinical setting, we must identify the individual MMPs that impact tumor progression in the bone. Our initial studies show that host MMP-9 does not contribute to tumor progression in the bone. In year 2, we will test the contribution of MMP-2, MMP-3 and MMP-13 to prostate tumor progression. While our initial observations with host MMP-9 have been negative, these data are important since they will allow for the development of selective MMP inhibitors. We hypothesize that selective MMP inhibitors will lack the deleterious side effects observed with broad spectrum inhibitors and therefore would be applicable in the clinical setting.

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